

Cyanobacteria from Cape Verde Islands: a contribution to the diversity and biotechnological potential

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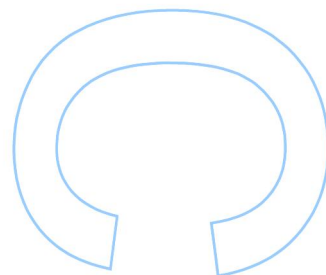
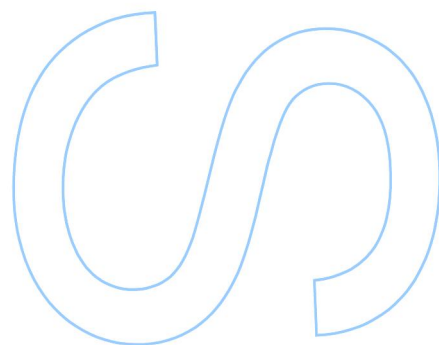
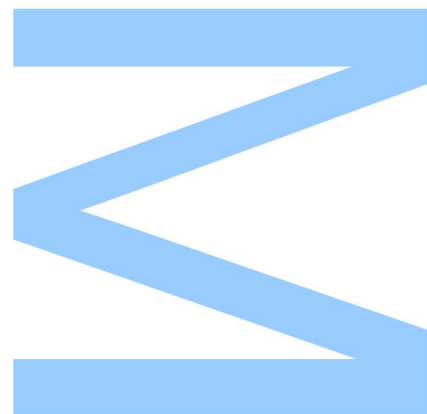
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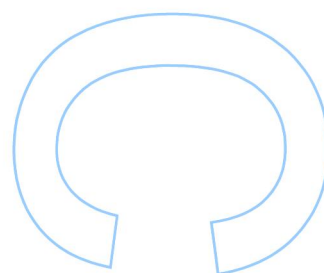
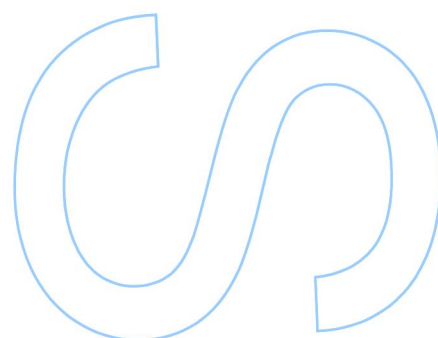
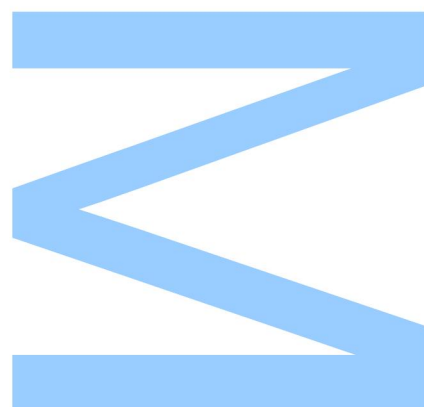
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Todas as correções determinadas
pelo júri, e só essas, foram efetuadas.
O Presidente do Júri,

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Abstract

Cyanobacteria are widespread prokaryotic photosynthetic organisms with an extreme capability to withstand different environment conditions. This adaptive capacity is attributed to the production of secondary compounds with antioxidant, photoprotective and humectant properties among others. In recent years marine cyanobacteria extracts and compounds revealed potential for biotechnology, namely in the pharmacological field. The isolation of marine cyanobacteria from different latitudes and its preservation *ex situ* is thus of crucial importance in order to trace their bioactive potential. In this work, coastal marine samples were collected from beaches of two Cape Verde Islands, Santo Antão and São Vicente. The objective was to contribute to the study of the cyanobacteria diversity of the archipelago and to isolate strains in order to infer about its biotechnological potential. From a total of 25 samples collected in São Vicente and 3 samples collected in Santo Antão, 20 cyanobacteria strains were isolated using both solid and liquid Z8 medium. Isolates were identified by morphometry and molecular tools, through the sequencing of the 16S rRNA gene. All the isolated strains are filamentous non-heterocystous forms. The phylogenetic analysis reveals strains that might belong to new genera, which contributes to enrich the diversity that occurs within this group of organisms.

To infer about the bioactive potential, strains were cultured in large scale for biomass production and methanolic extracts were prepared and tested for cytotoxicity using the normal keratinocytes cell line HaCat, the normal endothelial cell line hCMEC/D3, the hepatic carcinoma cell line HepG2, the colon carcinoma cell line RKO and the breast carcinoma cell line T47D. Cellular viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 24 and 48 hours of exposure to the extracts at a final concentration of 100, 75, 50, 25, 12,5 and 6,25 $\mu\text{g mL}^{-1}$. The MTT results revealed both toxic and no toxic responses revealing strains with potential for further fractionation. Among those the strain JM1B and JM5A induced selective cytotoxicity against the T47D cell line, revealing potential as anticancer. The strains PL1 and PL2 induced selective toxicity against the keratinocytes cell line, while strains VV5, VV3, VV3B and VV13 induced a proliferation of the same cell line. In both cases results revealed the potential of the strains for skin care applications.

Key words: Cape Verde; Cyanobacteria, Diversity; Natural compounds; Bioactivity.

Resumo

As cianobactérias são organismos procarióticos fotossintéticos com uma capacidade extrema de suportar diferentes condições ambientais. Essa capacidade adaptativa é atribuída à produção de compostos secundários com propriedades antioxidantes, fotoprotetoras e hidratantes, entre outras. Nos últimos anos, os extratos e compostos de cianobactérias marinhas têm revelado potencial para aplicação em biotecnologia, principalmente no campo farmacológico. O isolamento de cianobactérias marinhas de diferentes latitudes e sua preservação ex situ reveste-se assim de grande importância no sentido de avaliar o seu potencial bioativo. Este trabalho teve como base amostras recolhidas de zonas costeiras de duas ilhas de Cabo Verde, Santo Antão e São Vicente. O objetivo foi contribuir para o estudo da diversidade de cianobactérias do arquipélago e isolar estirpes para estudo sobre seu potencial biotecnológico. De um total de 25 amostras recolhidas em São Vicente e 3 amostras recolhidas em Santo Antão, conseguiu-se o isolamento de 20 estirpes usando meio Z8 sólido e líquido. Os isolados foram identificados por morfometria e ferramentas moleculares, através da sequenciação do gene 16S rRNA. Todas as estirpes isoladas são formas filamentosas não formadoras de heterocistos. A análise filogenética revela estirpes que podem pertencer a novos géneros, o que contribui para enriquecer a diversidade que ocorre dentro deste grupo de organismos.

Para avaliação do potencial bioativo, os isolados foram cultivados em larga escala para produção de biomassa e preparados extratos metanólicos. Os extratos foram testados quanto à citotoxicidade usando a linha celular normal de queratinócitos HaCat, a linha celular endotelial normal hCMEC / D3, a linha celular de carcinoma hepático HepG2, a linha celular RKO de carcinoma do cólon e linha celular T47D de carcinoma da mama. A viabilidade celular foi avaliada pelo ensaio de brometo de 3- (4,5-dimetiltiazol-2-il) - 2,5-difeniltetrazólio (MTT) após 24 e 48 horas de exposição aos extratos, numa concentração final de 100, 75, 50, 25, 12, 5 e 6,25 $\mu\text{g mL}^{-1}$. Os resultados do MTT revelaram respostas de citotoxicidade e proliferação seletiva, permitindo a seleção de algumas estirpes para posterior fracionamento. Entre estas, as estirpes JM1B e JM5A induziram citotoxicidade seletiva contra a linhagem de células T47D, revelando potencial anticancerígeno. As estirpes PL1 e PL2 induziram toxicidade seletiva contra a linha celular de queratinócitos, enquanto as estirpes VV5, VV3, VV3B e VV13 induziram uma proliferação da mesma linha celular. Nos dois casos, os resultados revelam o potencial das estirpes para aplicações cutâneas.

Palavras chave: Cabo Verde; Cianobactérias; Diversidade; Compostos naturais; Bioatividade.

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List of Abbreviations

ATCC - American Type Culture Collection

BBE – Blue Biotechnology Ecotoxicology

BLASTn-Basic Local Alignment Search Tool for nucleotides

CIIMAR - Interdisciplinary Centre of Marine and Environmental Research

CVA - Cape Verde Archipelago

DMEM Glutamax – Glutamax Dulbecco's Modified Eagle Medium

DMSO - Dimethyl sulfoxide

dNTPs - Deoxynucleotides

gDNA-Genomic deoxyribonucleic acid

EDTA Ethylenediamine tetraacetic acid

HaCat – Human keratinocytes cell line

hCMEC/D3 – Human endothelial cell line

HepG2-Liver hepatocellular carcinoma cell line

LEGEcc Blue Biotechnology and Ecotoxicology Culture Collection

MAAs-Mycosporine-like amino acids

MTT - 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide

MgCl₂ - Magnesium dichloride

NCBI - National Center for Biotechnology Information

PCR -Polymerase Chain Reaction

PBS - Phosphate-buffered saline

16S rRNA- RNA ribosomal 16S

RKO-Colon carcinoma cell line

SCY - Scytonemin

T47D- Human ductal breast epithelial tumor cell line

1. Introduction

1.1. Cape Verde Archipelago

Cape Verde Archipelago (CVA) is a group of ten volcanic islands located in the Tropical Eastern Atlantic, around the African coast (Figure 1). The archipelago main islands are split into two groups composed by six northern Barlavento (windward) Isles and four southern Sotavento isles. The origins of the archipelago are thought to be associated with hotspot activity impinging on the old oceanic lithosphere. Together with the Canary Islands, Madeira and Azores, Cape Verde belong to the Macaronesia biogeographic region [1]. This region is an important transitional climatic zone that encompasses a large part of the eastern north Atlantic, which is influenced by the semi-permanent Azores high-pressure system, prevailing north-easterly trade winds and the surrounding ocean. The Cape Verde climate ranges from tropical dry to semi-desert, mainly governed by seasonal changes in the location of the Azores anticyclone, the Intertropical Convergence Zone and arid Atlantic air masses [2].

Cape Verde precipitation is limited, and typically confined to the raining season (August-October). The two major influences on Cape Verde precipitation are the African Monsoon and African Easterly waves. As such, decadal changes in Cape Verde precipitation mirror precipitation trends across the West African Sahel, as they share the same generation mechanism [2].

São Vicente, one of the Barlavento (windward) islands, has an extension of 227 km². The depth of the sea floor between São Vicente and Santa Luzia does not reach 50 m, whereas between São Vicente and Santo Antão it reaches 500 m. Its outline resembles that of a rhombus with a major (E–W) diagonal of about 24 km and a minor (N–S) diagonal of about 16 km [3].

Santo Antão is the most north-western island of the CVA and the second largest of the ten main islands, with an area of 779 km². Santo Antão consists of alkaline volcanic rocks that vary in composition from nefelinite and basanite to phonolite, as for many volcanic oceanic islands [4]. The island has a rugged relief with the highest point at 1979 m. North-easterly trade winds ensure a humid climate and high erosion rates in the north and north-western parts of Santo Antão, which has many deeply dissected valleys where older successions of composite lava flows and associated dyke swarms are well exposed.



Figure 1 - Map of Cape verde archipelago

1.2. Cyanobacteria

Cyanobacteria are prokaryotic organisms with photosynthetic capacity but that are able to use also anaerobic respiration and to fix atmospheric nitrogen. In the marine environment cyanobacteria are considered the main producer of the phytoplankton community food chain as they produce 50% of the biomass [5]. Also known as cyanophyta or blue-green algae, cyanobacteria have a great physiological and

morphological variability, which might be the base for their global distribution [6]. In fact, cyanobacteria have a wide distribution and can be found in aquatic environments as well as in terrestrial environments and even in extreme conditions such as a high salinity [7] high and

low temperature [8]. In order to survive to such extreme conditions through a long evolution period cyanobacteria have developed mechanisms of adaptation that include the production of sundry secondary metabolites [9], with antioxidant, photoprotective, moisturizing, allelopathic and toxic properties, which includes pigments, polysaccharides, fatty acids and peptides [10].

The bioactive properties of cyanobacteria, the high growth rates and the ability to grow autotrophically which makes their cultivation simple and cost-effective, have attracted an increasing interest by a wide range of industries, such as the food industry, the cosmetic industry, the pharmacological industry, the energetic industry and agriculture. In fact cyanobacteria have been considered a rich source of compounds for use as food additives, cosmetics, antitumor, antibacterial and antiviral drugs, biofuels as well as organic fertilizers [11].

The high interest in cyanobacteria has led to further research on strains from different habitats to obtain value-added strains for industry and agriculture. This research involves collecting samples in the wild, isolation, identification and culture. In order to maintain the isolated strains, biologic specimens must be deposited in collections, being then available for investigation.

1.2.1. Cyanobacteria taxonomy

The cyanobacterial phylum is very diversified in terms of morphology, including unicellular, colonial and filamentous forms. In some filamentous strains a cell differentiation is presented by the production of specialized cells involved in nitrogen fixation, the heterocysts, and resting cells that can resist to environmental stress, the akinetes. Some genera also produce small fragments with different size and shape, the hormogonia, used for dispersal and host infection [12].

Traditionally, cyanobacteria were classified according the International Code of Botanical Nomenclature due to the ability to perform oxygenic photosynthesis and the similarity to the structure of eukaryotic chloroplasts. However, due to their prokaryotic nature cyanobacteria are also classified according to the International Code of Nomenclature of Bacteria. Currently the two approaches prevail, the botanical [13] and the bacterial [14].

According to the botanical classification cyanobacteria are classified into five Orders while according to the Bacterial approach cyanobacteria are classified into five Subsections. The five orders and subsections broadly coincide as described in Table 1, where the main characteristics are presented.

Although the traditional classification and identification of cyanobacteria has been based on morphological characteristics, the advances in molecular biology led to the use of molecular markers. A combination of different approaches has thus been lately adopted, which includes not only the morphological description of the specimens but also a genotypic analysis, namely by using the small subunit ribosomal RNA, the 16S rRNA gene [15]. This new polyphasic approach makes it possible to overcome some difficulties in morphology-only identification due to the small size of many strains and morphological changes during strain cultivation or long-term maintenance in the laboratory [15].

Table 1 - Main characteristics and genus of the 5 orders/subsections of the phylum cyanobacteria.

Orders/ Subsections	Main characteristics	Genus
Chroococcales Subsection I	Unicellular or not aggregated. Reproduction by binary fission in one, two or more plans.	<i>Chroococcus</i> , <i>Cyanobacterium</i> , <i>Cyanothece</i> , <i>Dactylococcopsis</i> , <i>Gloeobacter</i> , <i>Gloeotheca</i> , <i>Microcystis</i> , <i>Prochlorococcus</i> , <i>Synechococcus</i> , <i>Synechocystis</i>
Pleurocapsales Subsection II	Unicellular. United by exterior walls or gel matrix. Reproduction by multiple fusion. The tilakoid cells are smaller than the stem cells.	<i>Cyanocystis</i> , <i>Dermocarpella</i> , <i>Myxosarcina</i> , <i>Stanieria</i> , <i>Xenococcus</i>
Oscillatoriales Subsection III	Filamentous. Reproduction by binary fission in one plan. Unbranched and uniseriate trichomes. Trichomes without heterocysts or akintes.	<i>Arthrospira</i> , <i>Lyngbya</i> , <i>Leptolyngbya</i> , <i>Microcoleus</i> , <i>Oscillatoria</i> , <i>Planktothrix</i> , <i>Pseudoanabaena</i> , <i>Trichodesmium</i>
Nostocales Subsection IV	Filamentous. Reproduction by binary fission in one plan. Unbranched and uniseriate trichomes. Trichomes with heterocysts or akintes.	<i>Anabaena</i> , <i>Anabaenopsis</i> , <i>Aphanizomenon</i> , <i>Cylindrospermopsis</i> , <i>Gloeotrichia</i> , <i>Nodularia</i> , <i>Nostoc</i> , <i>Rivularia</i> ,
Stigonematales Subsection V	Filamentous. Reproduction by binary fission periodically or in more than one plan. Multiseriated trichomes or trichomes with various types of branches. Trichomes with heterocysts or akintes.	<i>Loriella</i> , <i>Geitleria</i> , <i>Nostochopsis</i> , <i>Westiella</i>

1.2.2. Cyanobacteria isolation and preservation

Although for several years cyanobacteria were found to be difficult or even impossible to isolate, Allen (1968) and Huges (1958) proved that it was possible to isolate and maintain ex-situ cyanobacteria in laboratory culture, for several years [16],[17]. To initiate the process of cyanobacteria isolation, environmental samples are collected, and their origin noted. Upon arrival to the laboratory, it is critical to evaluate the sample material content in order to identify cyanobacteria that have capacity to grow in specific medium, since the characteristics that an organism must develop in a given environment are used as selective factors in the isolation process. This evaluation is carefully performed with the aid of a microscope [18]. After, it is crucial the choice of the most appropriate medium to their successful isolation. The medium used for enrichment is generally liquid and have a nutritional composition that enables the development of specific organisms to the detriment of competitors. These media may be selective enrichment media, if it involves the addition of inhibitory substances (eg antibiotics, antifungals) that suppress the growth of most organisms with the exception of the organism to be isolated [19]. Following the collection, identification, and characterization of organisms, environmental samples should be enriched with the selected medium.

In addition to isolation in liquid media, cyanobacteria can also be isolated using solid media such by inoculum streak and streak seeding [18] or selecting organisms by micromanipulation, a technique that requires practice and patience [20], or a combination of these different techniques. The micromanipulation is performed with the aid of an optical microscope and consists of the selection of the organism of interest, ideally present in liquid medium, using microcapillaries [20]. The obtained cultures should be monitored and successively picked until isolation is confirmed [20].

After isolation and classification, strains must be preserved in controlled culture conditions in order to ensure the ex-situ preservation. This ex-situ preservation of microorganisms and the public access to microbial specimens is the aim of the microbial domain of *Biological Resource Centres* (mBRC), the so-called microbial Culture Collections.

Blue Biotechnology and Ecotoxicology (BBE) Culture Collection (LEGE-CC) <http://lege.ciimar.up.pt/> is a biological resource center of the Interdisciplinary Marine and Environmental Research Center (CIIMAR) Portugal. The collection currently comprises about 1000 of cyanobacteria and microalgae strains collected from different sites, mainly in mainland Portugal including Madeira and Azores Islands, but also elsewhere (e.g. Australia, Brazil, Colombia, Morocco, Mexico). LEGE CC strains are derived from many

environments, 93% of those are from aquatic environments (2% hypersaline, 46% marine, 11% brackish and 34% freshwater), 3% from terrestrial environments and 4% with unknown origin. They are also producers of secondary metabolites, such as microcystin, that have toxic effects on human beings [22]. One of the main purposes of LEGE-CC is to provide living organisms, and/or related information, to third parties for use in research and development activities. It is therefore essential that strains existing (or to be included) in the LEGE-CC crop collection are properly isolated and appropriately characterized in order to interest potential users [21].

1.2.3. Cyanobacteria bioactive compounds

A main reason why the study of cyanobacteria is so attractable is the production of bioactive compounds with diverse biotechnological applications. Cyanobacteria bioactive compounds are mainly secondary metabolites. These are metabolites produced by an organism in which a role in primary functions such as growth or reproduction has not been found [23]. They are responsible for umpteen functions such as allelopathy [24] and defense [25], although they are not absolutely required for the survival of the organism.

The high biodiversity of cyanobacteria in nature is reflected also in a high chemical diversity and bioactive potential. The range of bioactive secondary compounds from cyanobacteria includes alkaloids, peptides, lipopeptides, amino acids and fatty acids [26], being cyanobacteria considered a good source of compounds for biotechnology applications, namely for pharmaceutical and therapeutic application [27],[28],[29],[30]. In fact, in the last few years cyanobacteria have gained a lot of attention being considered one of the most promising sources of natural compounds with antibacterial [31], antiviral [32], antifungal, anti-inflammatory, algicide [33], anticancer [34, 35], and antiparasitic activity [26] (Table 2)

Table 2 - Marine cyanobacteria compounds with bioactive potential (anticancer, anti-inflammatory, antioxidant, antiparasitic, photoprotective and whitening).

	Compound or extract	Source	Class of compound	Biological Model	Reference
Anticancer	Lagunamides	<i>Lyngbya majuscula</i>	Cyclic depsipeptide	Cytotoxicity against a panel of cancer cell lines	[36]
	Aurilides	<i>Lyngbya majuscula</i>	Cyclic depsipeptide	Cytotoxicity against NCI-460 and neuro-2a mouse neuroblastoma cells	[37]
	Isomalyngamide	<i>Lyngbya majuscula</i>	Fatty acid amides	Activity against tumor cell migration through the 1 integrin-mediated antimetastatic pathway	[38]
	Palmyramide A	<i>Lyngbya majuscula</i>	Cyclic depsipeptide	Blocking of sodium channels in neuro-2a cells and cytotoxicity against H460 human lung cancer cells	[39]
	Biselyngbyaside	<i>Lyngbya sp</i>	Glicomacrolide	Cytotoxicity against a panel of cancer cell lines	[40]
	Bisebromoamide	<i>Lyngbya sp</i>	Peptide	Cytotoxicity against a panel of cancer cell lines	[41]
	Apratoxins	<i>Lyngbya</i>	Cyclic Depsipeptide	Cytotoxicity against a panel of cancer cell line	[42, 43]
Anti-inflammatory	Sacran	<i>Aphanothece sacrum</i>	Polysaccharide	Mice	[44]
Antioxidant	Scytonemina (SCY)	<i>Scytonema sp.</i>	Alkaloid	Cyanobacteria cells	[45]
	Mycosporine-like amino acids (MAA)	<i>Nostoc sphaericum</i>	-	Cyanobacteria cells	[46]
	MAAs and SCY	<i>Nostoc commune</i>	-	Cyanobacteria cells	[47]
	Exopolysaccharides (EPS)	<i>Nostoc commune.</i>	Polysaccharides	Caenorhadiris elegants	[48]

Table 2 (Cont.)

	Compound or extract	Source	Class of compound	Biological Model	Reference
Antiparasitic (Leishmania)	Venturamide B	<i>Oscillatoria sp.</i>	Cyclic peptides	<i>Leishmania donovani</i>	[49]
	Gallinamide A	<i>Schizothrix sp.</i>	Peptide	<i>L. donovani</i>	[49]
Antiparasitic (Malaria)	Lagunamide A	<i>Lyngbya majuscula</i>	Cyclic depsipeptides	<i>Plasmodium falciparum</i>	[50]
	Venturamide A	<i>Oscillatoria sp</i>	Cyclic hexapeptides	<i>P. falciparum</i>	[51]
Antiparasitic (Tripanossoma)	Venturamide B	<i>Oscillatoria sp.</i>	Cyclic peptides	<i>Tripanosoma. Cruzi</i>	[49]
	Dragonamide E	<i>Lyngbya majuscula</i>	Lipopeptides	<i>L. donovani</i>	[52]
Photo Protection	SCY	<i>Arthrospira platensis</i>	Alkaloid	Cyanobacterial cells	[45]
	MAAs and SCY	<i>Lyngbya sp.</i>	—	Cyanobacterial cells	[53]
Whitening	Chloroform-methanol extract	<i>Oscilnorlatoria agardhii</i>	-	Tyrosinase activity assay	[54]
	C-phycocyanin	<i>Spirulina</i>	Pigment	B16F10	[55]

2.Objectives

The main aim of this work was to contribute to the study of the biodiversity and the biotechnological potential of cyanobacteria collected from Cape Verde Islands. The environmental samples included in this study were collected from two islands of the archipelago, São Vicente and Santo Antão. Samples were collected by members of the Blue Biotechnology and Ecotoxicology Group (BBE) from the Centre of Marine and Environmental Research (CIIMAR).

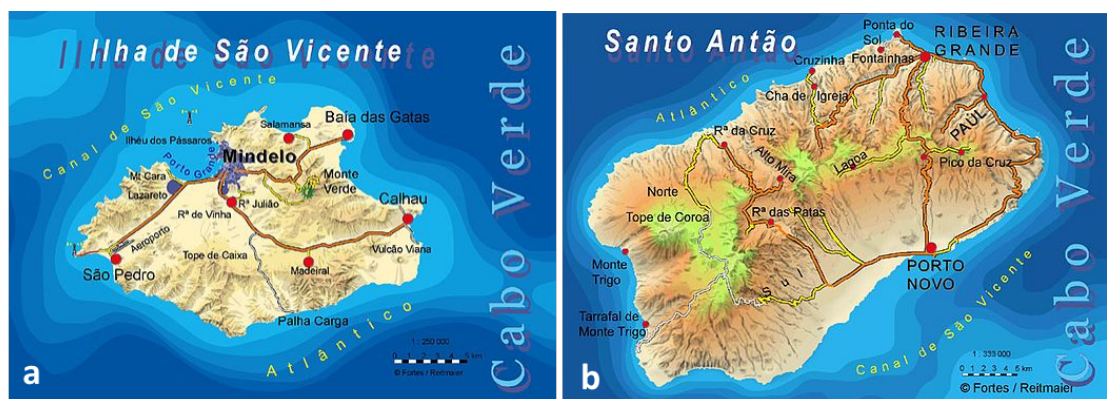
Based on this main goal, specific tasks were performed, which included:

1. Isolation of cyanobacteria strains using both solid and liquid Z8 medium;
2. Identification of the isolated cyanobacteria using a morphological and a molecular approach;
3. Culture of isolated cyanobacteria strains for biomass production.
4. Preparation of cyanobacteria extracts;
5. Evaluation of the bioactive potential of cyanobacteria extracts:
 - a. evaluation of the anticancer potential using cancer cell lines and normal cell lines.

3. Material and Methods

3.1. Sampling

The biological environmental samples were collected in April 2018 in two Cape Verde Islands, São Vicente and Santo Antão. In São Vicente, samples were collected in Baía das Gatas, Praia da Laginha, Cova da Inglesa, Salamansa and Calhau. In Santo Antão samples were collected in Ponta do Sol (Figure 2; Table 1).



(https://en.wikipedia.org/wiki/S%C3%A3o_Vicente,_Cape_Verde; https://en.wikipedia.org/wiki/Santo_Ant%C3%A3o,_Cape_Verde)

Figure 2 - Samples locations in São Vicente (Baía das Gatas, Praia da Laginha, Cova da Inglesa, Salamansa, Calhau) and Santo Antão (Ponta do Sol).

The collected material consisted in rock scrapes along the coast and sea water samples. After collection the material was stored in thermal bags and kept refrigerated until processing.

Table 3 - Sampling sites and environmental samples collected from São Vicente and Santo Antão islands (data kindly provided by João Morais, BBE-CIIMAR).

Island	Site	Sampling Date	Original label
São Vicente	Baía das Gatas	14/04/2018 and 20/04/2018	JM1
	Baía das Gatas	14/04/2018	JM2
	Baía das Gatas	14/04/2018	VR1
	Baía das Gatas	14/04/2018 and 20/04/2018	VR2
	Baía das Gatas	14/04/2018	VR3
	Baía das Gatas	14/04/2018	VV1
	Baía das Gatas	14/04/2018	PL1
	Baía das Gatas	14/04/2018	PL2
	Baía das Gatas	14/04/2018	PL3
	Calhau	24/04/2018	VV8
	Calhau	24/04/2018	VV9
	Calhau	24/04/2018	VV10
	Cova da Inglesa (Lazareto)	21/04/2018	VV5
	Cova da Inglesa (Lazareto)	21/04/2018	JM3
	Cova da Inglesa (Lazareto)	21/04/2018	JM4
	Cova da Inglesa (Lazareto)	21/04/2018	JM5
	Praia da Laginha	21/04/2018	VV2
	Praia da Laginha	21/04/2018	VV3
	Praia da Laginha	21/04/2018	VV4
	Salamansa	23/04/2018	VV6
	Salamansa	23/04/2018	VR4
	Salamansa	23/04/2018	VV7
	Salamansa	23/04/2018	VR5
	Salamansa	23/04/2018	VR6
	Salamansa	23/04/2018	VR7
Santo Antão	Ponta do Sol	25/04/2018	VV11
	Ponta do Sol	25/04/2018	VV12
	Ponta do Sol	25/04/2018	VV13

3.2. Isolation of cyanobacteria strains

The isolation of cyanobacteria from environmental samples was performed using both liquid and solid Z8 culture medium, supplemented with 25g/L NaCl [56], in aseptic conditions. All the glass material used was autoclaved (Tuttnauer 3870 ELV-D, The Netherlands) for 20 min at 121°C. The preparation of the medium and the entire isolation process was carried out in a laminar flow chamber (Telstar Bio II Advance, Spain) previously disinfected with 70% alcohol and exposed to UV light for 30 minutes before each use.

For solid culture medium, agarose was added to Z8 medium to a concentration of 1,2%. The mixture was spread on sterile polystyrene Petri dishes and after solidification, the Petri dishes were stored aseptically for further use.

The solid Z8 medium was the primary method used to isolate cyanobacteria from the environmental samples. All the media for the raw environmental samples were prepared with cycloheximide, at a concentration of 0.025%, to prevent the growth of eukaryotic microorganisms.

3.3. Identification of cyanobacteria strains

3.3.1. Morphological characterization

To morphologically characterize the isolates, strains were observed, annotated and photographed using an optical microscope (Leica DMLB) coupled to a camera (Leica ICC50 HD) with the Leica Qwin Color computer program (Leitz, Germany) at a magnification of 1000X. For each isolate, morphometric measurements were also made and the mean dimensions (length, width) of the vegetative cells of filaments recorded. Length and width were measured in 20 cells, being the cells from different individuals of each strain.

3.3.2. Molecular characterization – DNA extraction and PCR conditions

3.3.2.1. DNA extraction

To extract the gDNA from the isolated strains, a small amount of biomass was collected in 1.5 mL Eppendorf tubes, in the flow chamber. Tubes were centrifuged at 10000 X g for 1 minute to concentrate the biomass and remove the liquid in excess. The biomass was then stored at -20 °C for posterior extraction of DNA.

Extraction of gDNA was performed using the Genetic DNA Mini-Kit Purelink (Invitrogen, USA), using the protocol for extracting DNA from gram-negative bacterial cells, according to the manufacturer's instructions. Isolated DNA was stored in 1.5 mL Eppendorf tubes at a temperature of -20 ° C.

To confirm that the DNA was successfully extracted an electrophoresis in Agarose gel (Ultrapure™ Agarose, Invitrogen, USA) at 1% concentration in Tris-Acetate EDTA buffer solution (TAE Ultrapuretm, Invitrogen, USA) at 40mM Tris-acetate and 1mM EDTA, was performed. Gel was stained with 3µL of SYBRsafe (Invitrogen, USA).

Briefly, 3 µl of extracted DNA mixed with 0.5 µl of loading buffer were loaded onto the gel, 1 µl molecular marker (1Kb Plus DNA Ladder-Invitrogen) was loaded. The gel was run at 85V for 20 minutes and visualized and photographed on the GEL DOCTM Molecular Imager® transilluminator with Image Lab™ software (USA).

3.3.2.2. PCR amplification and analysis of products

The molecular identification of isolates was based on the 16S rRNA gene. Information on primers is described in Table 4.

Table 4 - Primer pairs used for amplification of the 16S rRNA gene

Target gene	Primer	Primer sequence (5' _3')	Target Group	size (bp)	Reference
16S rRNA	27F	AGAGTTTGATCCTGGCTCAG	Cyanobacteria	754bp	[57]
	781R	GACTACWGGGGTATCTAATC CCWTT			[58]
	1494R	TACGGCTACCTTGTTACGAC		1135bp	[58]

	359F	GGGGAATYTTCCGCAATGGG			[57]
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PCR reactions consisted in 1x GoTaq buffer, 2.5 mM for MgCl₂, 1 µM for each primer, 0.5 mM for the dNTP mix, 0.5 U for GoTaqR Flexi DNA polymerase. The final volume of each reaction was 20 µL with 1 µL of DNA template (Table 5).

Table 5 - Mastermix components of the PCR reaction

Components	Volume per reaction (1x)
Molecular biology water	7.9 µL
5x Buffer	4 µL
MgCl ₂	2 µL
Forward primer	2 µL
Reverse primer	2 µL
Deoxynucleotides (dNTP's)	2 µL
Taq Polymerase	0.1 µL
DNA template	1 µL
Total:	20 µL

The PCR reactions were performed in a Biometra T-Professional Standard Gradient Thermocycler (Germany) as described in table 6.

Table 6 - PCR conditions used for the primer pair used to amplify the 16S rRNA gene

Target gene	Primer Pair	PCR reaction					References
		Initial Denaturation	PCR cycles			Final extension	
RNA 16S	27F/359F	95°C 2 mn	35 Cycles			72 °C 5 mn	[59]
	781R/1494R		95°C 2 mn	55°C 45 s	72°C 1 mn		

After amplification, all PCR products were analyzed and visualized via electrophoresis in an agarose gel ((Ultrapuretm Agarose, Invitrogen, USA) at a concentration of 1.5% which was stained with 3µL of SYBRsafe (Invitrogen, USA). The selected voltage and running time were 85 V and 20 minutes. The agarose gel was visualized and photographed in

the transilluminator Molecular Imager® GEL DOCTM with the software Image LabTM(USA).

3.3.2.3. Sequencing, sequence alignment and phylogenetic analysis

All the samples that exhibited positive PCR results (i.e presence of the gene) were selected for sequencing. The 16S rRNA gene was firstly amplified and sequenced for all the isolates obtained, in order to identify the cyanobacteria and thus assess the diversity of the samples. For sequencing purposes, the PCR preparation involved duplicating the number of reactions for each isolated sample, in order to have enough amplified product to enable sequencing. So, the final volume of PCR product per sample loaded into the 1.5% agarose gel was 40 µL. The electrophoresis was run at 90 V for 60 minutes, then the amplified fragments were observed in the CSMICRODOC system (Cleaver scientific, UK) transilluminator coupled with a Canon PowerShot G9 camera. The bands with the expected size were excised from the gel and collected to be purified using the Nzttech – genes & enzymes (NZYGelpure, Portugal) purification kit, following the manufacturer's instructions. To check the efficacy of the DNA purification, an electrophoresis (100V; 20 minutes) was run in a 1% agarose gel, and 2 µL of the purified DNA was mixed with 1 µL of loading buffer. All the purified PCR products and the respective pair of primers was sent to GATC Biotech (Germany) to be sequenced.

The forward and reverse sequences (i.e. 5' and 3') obtained from the same PCR product were examined in the bioinformatic software Geneious (v.8) and assembled together (de novo assembly), their chromatograms were analyzed to check the quality of the sequences and to determine if further sequences were needed to form a consensus sequence (i.g. if the quality was bad on either one of the forward or reverse sequences). The sequences usually be trimmed at the extremities due to bad quality. Then, the consensus sequences of each strain isolate were compared with the sequences in the GenBank® database using the BLAST®n (Basic Local Alignment Search Tool for nucleotides) tool available in the NCBI (National Center for Biotechnology Information) and compared with other cyanobacterial sequences in the GenBank® database to check for similarities and to help in their identification. To settle the phylogenetic relation of the isolated strains to other reference strains, the sequences were aligned by ClustalW, and the GTR+G+I evolutionary model was selected by MEGA 7.0. The robustness of

Maximum Likelihood (ML) trees was estimated by bootstrap percentages, using 1000 replications in MEGA 7.0.

3.4. Cyanobacteria culture and extraction

For stock and culture, the cyanobacteria strains were inoculated in 500 mL and 4 liters of Z8 medium (with 25g/L NaCl) respectively. Cultures in 4L medium were maintained under an aeration system. All the procedures were performed in controlled temperature conditions (25 °C) and luminance (light cycles / dark 14h / 10h). When cultures reached the exponential growth stage, after approximately 30 days of the inoculation, visually recognized by the high concentration of biomass (Figure 2), the cultures were harvested by filtration.



Figure 3 - Cyanobacteria cultures after 25 days growth.

Before lyophilization, the concentrated biomass was stored at -80°C . The frozen biomass was lyophilized for 7 days at -48°C (Telstar LyoQuest) under reduced pressure (0.1 mbar with condenser at -47°C) and stored at -20°C until further use.

A smaller stock culture of all cyanobacteria strains was maintained during this work in order to provide biomass to inoculate in the 500 mL medium. This stock culture was

performed by removing a small amount of biomass of the culture and placing it in flasks with 100 ml of Z8 medium (25g/L NaCl).

The cyanobacterial extracts were performed with lyophilized material. The extraction was started by breaking the lyophilized biomass using a mortar and porcelain pestle, in small portions at a time, transferring the grounded biomass to an Erlenmeyer flask. After, 50 ml of methanol were added, and the flask placed in the ultrasonic bath for 5 minutes (taking care not to heat above 30 °C). The flask was rested for a few minutes, letting the particulate matter settle to the bottom. The supernatant was filtered using a vacuum filtering system as describe in the Fig. 4. This procedure was repeated twice using 25 mL of methanol. The final extract was concentrated in a round flask on a rotary evaporator at 30 °C, under a control pressure. The concentrated extract was transferred to a pre-weighed 20 mL vial, by filtering it through a Pasteur pipette with a cotton filter (to capture any non-soluble material). Finally, the solvent was evaporated in a rotary evaporator and the flask was left in high vacuum to thoroughly dry. The glass vial was weighted again, and the total mass of the crude extract calculated. The dry extract was dissolved at the concentration of 10 mg/mL using DMSO and stored at -20 °C.

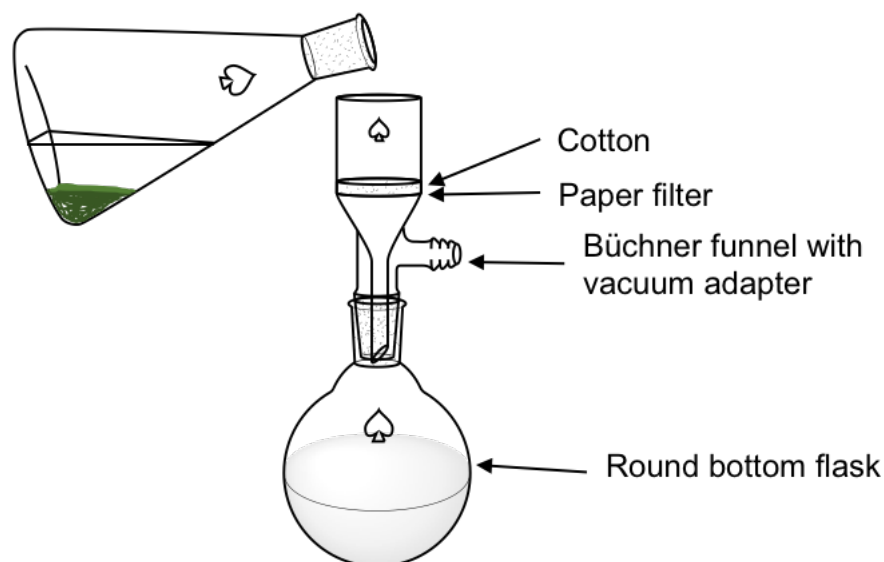


Figure 4 - Extraction system from lyophilized cyanobacterial material (according to the MeOH extraction SOP V12.2019 protocol from BBE.CIIMAR).

3.5. Cytotoxicity analysis

3.5.1 Cell culture

The bioactivity of the extracts was assessed using normal and cancer cell lines (Table 7).

Table 7 - Cell lines included in the study

Cell	Cell line	Origin
Hepatic carcinoma cells	HEPG2	American Type Culture Collection (ATCC)
Colon carcinoma cells	RKO	American Type Culture Collection (ATCC)
Breast adenocarcinoma cells	T47D	Sigma-Aldrich
Keratinocytes	HaCaT	American Type Culture Collection (ATCC)
Endothelial cells	hCMEC/D3	Donated by Dr. PO Couraud (INSERM, France)

Cells were cultured in DMEM Glutamax medium (Dulbecco's Modified Eagle Medium DMEM GlutaMAX™ – Merck, Germany), supplemented with 10% (v/v) fetal bovine serum (Gibco, USA), 0,1% Amphotericin B (Gibco, Germany) and 1% of penicillin-streptomycin (Pen-Strep 100 IU/ml and 10 mg/ml, respectively) (Gibco, Germany) in a humidified atmosphere containing 5% CO₂ and at 37°C. Culture medium was renewed every two days. At 80-90% cell confluence, adherent cells were washed with phosphate-buffered saline (PBS) (Gibco, Germany) and enzymatically released with TrypLE express enzyme (1x) (Gibco, Denmark).

3.5.2 Cytotoxicity assay – MTT assay

The viability of cells exposed to the cyanobacteria extracts was evaluated by using the 3- (4,5 dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT is

a yellow tetrazole soluble in water that is reduced by mitochondrial enzymes to purple formazan crystals that are insoluble in water. The reduction of MTT to formazan is directly proportional to the mitochondrial activity and consequently cell viability ([61].

For the cytotoxic assays cells were seeded in 96-well culture plates at a density of $2,5 \times 10^4$ cells mL^{-1} for keratinocytes, 1×10^5 cells mL^{-1} for endothelial cells and 3.3×10^4 cells mL^{-1} for T47D, RKO and HepG2 cells. After 24 hours of adhesion, cells were exposed to 100 μL fresh medium supplemented with the extracts to a final concentration of 100, 75, 50, 25, 12,5 and 6,25 $\mu\text{g mL}^{-1}$ for a period of 24 and 48 hours. After each incubation time, an aliquot of 20 μL MTT solution (1 mg mL^{-1}) was added to each well in medium and incubated at 37°C for 3h. After the incubation time, the medium was carefully aspirated, and the purple colored formazan salts dissolved in 100 μL DMSO. Absorbance was read at 550 nm in using a Synergy HT Multi-detection microplate reader (Biotek, Bad Friedrichshall, Germany) operating by GEN5™ software.

4. Results and Discussion

4.1. Isolated strains and identification

The isolation procedure, as well as time consuming, does not isolate all the diversity in a sample collected at a given location. A total of twenty isolates were obtained (Table 8 and Figure 5). All the isolated were obtained by petri dish spreading. From the total, nineteen strains were isolated from São Vicente island and one strain was isolated from Santo Antão. Due to time constraints deadlines only Z8 medium was used. Although Z8 medium is a nutrient rich medium and widely used in the isolation of cyanobacteria, it may not have facilitated the isolation of some groups, namely coccoid forms and heterocystous forms. In this sense other media may be used in the continuation of the work, such as culture medium BG11₀.

For the identification of the isolates, a polyphasic approach was followed. These included morphological characteristics such as the presence of specialized cells and cell size, and molecular tools such as the amplification and sequencing of the 16S rRNA gene [62].

As we can see in the Table 8 the morphological diversity of cyanobacteria is quite variable, however, it is verified that the isolates obtained from different places of the Islands have some peculiar and distinct characteristics. All the isolates are filamentous and no specialized cells such as heterocysts are present. This might be due to the faster growth occurring in Z8 medium, which seemed to favor the growth of non-heterocystous strains.

There are isolates that have filaments with rounded end cells, others that have slightly arched filaments at the ends and other cellular constrictions at wall level. Still others have thin filaments that coil, some in the shape of a ball. The isolates color ranged from green, dark green, brownish to reddish. By the color it was possible to conclude that the strains JM1C, VV2, VV3 and VV5 are rich in phycoerythrin responsible for the reddish color (Figure 5). These may be interesting to produce the red pigment that can potential be use as a natural colorant for the food and cosmetic industry.

Table 8 - Isolated strains, morphological characteristics.

Sampling site	Original Label	Isolate code	Color in culture	Morphological characteristics	Cell size (Width x Length) µm
São Vicente					
Baia das Gatas	JM1	JM1B	Cyano	Filaments with sheath	1,2±0,1x1,3±0,3
		JMIC	Reddish	Filaments with trichomes constricted at cross walls	1,6±0,2 x 1,6±0,3
B. das Gatas	PL1	PL1	Cyano	Filaments with sheath and terminal cells rounded	2,4±0,5 x 2,7±0,8
B. das Gatas	PL2	PL2	Cyano	Filaments with sheath	2,3±0,5 x 4,7±1,4
B. das Gatas	PL3	PL3	Cyano	Filaments with sheath	2,3±0,2 X 3,0±1,0
B. das Gatas	VV1	VV1	Cyano	Filaments with sheath	1,4±0,2 x 1,4±0,2
Calhau	VV9	VV9	Dark brown	Filaments with some helical filaments, forming tuft like aggregates	1,8±0,2 x 1,5±0,3
Calhau	VV10	VV10	Cyano	Thin, coiled filaments wrapped in mucilage	1,1±0,2 x 1,4 ±0,4
Cova da Inglesa	VV5	VV5	Brownish	Filaments growing homogeneously, but also forming aggregates	1,5±0,3 x 1,5±0,3
C. da Inglesa	JM5	JM5A	Cyano	Filaments with sheath	1,2±0,3 X 1,1±0,2
Praia da Laginha	VV2	VV2	Reddish	Trichomes slightly constricted at cross walls	1,8±0,3 X 1,4±0,3
P. da Laginha	VV3	VV3	Reddish	Thin, coiled filaments wrapped in mucilage	1,2±0,3 x 1,2±0,3
		VV3A	Green olive	Filaments growing homogeneously, but also forming aggregates	1,4±0,2 x 1,4±0,2
		VV3B	Dark green	Filaments growing homogeneously, but also forming aggregates	1,1±0,18 x 1,2±0,3
P. da Laginha	VV4	VV4	Reddish	Trichomes slightly constricted at cross walls	1,7± 0,3 X 1,6±0,2
Salamansa	VR5	VR5	Cyano	Filaments forming a smooth biofilm	1,1± 0,2 x 1,2±0,2
Salamansa	VV6	VV6	Cyano	Filaments growing homogeneously, but also forming aggregates	1,2±0,1 x 1,4±0,3
Salamansa	VR7	VR7A	Cyano	Filaments growing homogeneously also forming aggregates	1,6±0,3 x 1,6±0,4
		VR7B	Dark green	Thin, coiled filaments wrapped in mucilage	1,3±0,3 x 2,2±1,0
Santo Antão					
Ponta do Sol	VV13	VV13	Reddish Brawn	Filaments with sheath and rounded cells rounded	1,9±0,4 x 1,7±0,5

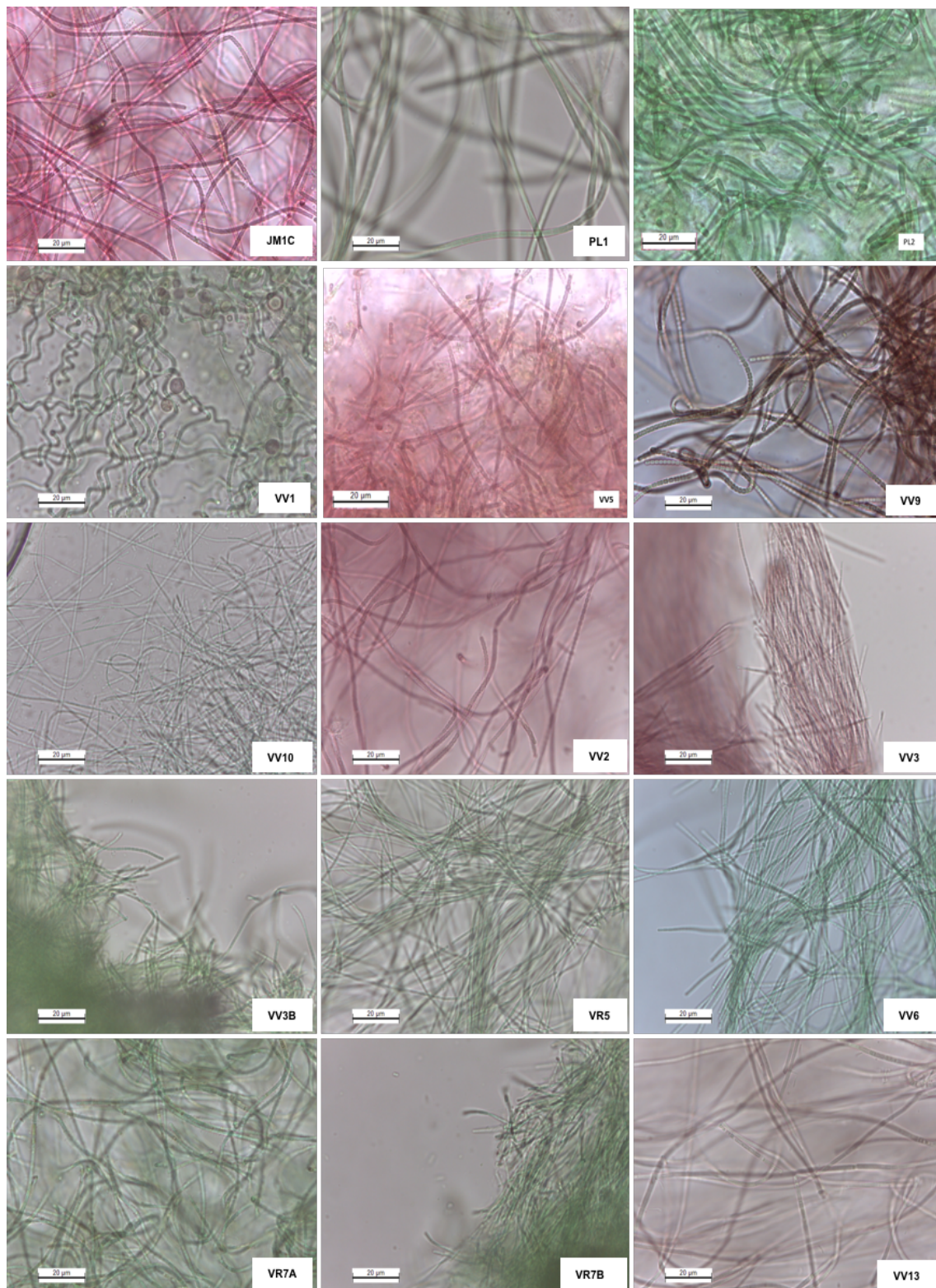


Figure 5 - Photomicrographs of the cyanobacteria isolates.

4.2. Phylogenetic analysis

The identification of the cyanobacteria isolates by a molecular approach was based in the 16S rRNA gene sequences. In Table 9 it is presented the best BLAST results obtained from Genbank for the consensus sequence of sixteen of the isolates. The sequences of the BLAST results from the isolates of this study and a subset of 56 sequences representing cyanobacterial diversity (obtained from the online database Cyanotype) were integrated into the phylogenetic tree shown in Figure 6.

Table 9 - Best Blast results obtained for each isolate based on the molecular marker 16S rRNA

Isolate Code	Molecular Identification (ID) (top cyano hit)	% ID	Sequence Length bp
JM1B	Filamentous cyanobacterium ESFC-1 clone 4_5A 16S ribosomal RNA gene	96.45%	733
JM1C	Leptolyngbya sp. RS02 16S ribosomal RNA gene	99.8%	1363
PL1	Oscillatoria sp. S8 16S ribosomal RNA gene	99.72%	1053
VV1	Nodosilinea sp. LEGE 11468 16S ribosomal RNA gene	99.62%	1330
PL2	Geitlerinema sp. PCC 7105 small subunit ribosomal RNA gene, partial sequence	99.49%	1369
VV9	Leptolyngbya sp. PCC 7376, complete genome	99.26%	1355
VV10	Filamentous cyanobacterium 73-2 16S ribosomal RNA gene	97.69%	1382
JM5A	Nodosilinea sp. LEGE 11468 16S ribosomal RNA gene	99.26%	1079
VV2	Leptolyngbya sp. RS02 16S ribosomal RNA gene, partial sequence	99.22%	1279
VV3	Phormidium angustissimum SABC020801 16S ribosomal RNA gene	96.71%	1370
VV3A	Filamentous cyanobacterium 73-2 16S ribosomal RNA gene	99.98%	1372
VV3B	Filamentous cyanobacterium 73-2 16S ribosomal RNA gene	99.13%	1375
VV6	Filamentous cyanobacterium 73-2 16S ribosomal RNA gene	99.13%	1380
VR7A	Filamentous cyanobacterium 73-2 16S ribosomal RNA gene	99.12%	1366
VR7B	Nodosilinea sp. LEGE 11468 16S ribosomal RNA gene, partial sequence	99.62%	1042
VV13	Leptolyngbya ectocarpi LEGE 11474 16S ribosomal RNA gene	97.73%	1189

In the total of 20 isolated strains, 16 could be analyzed through molecular tools while for VV5, VR5, PL3 and VV4 only morphological characteristics were able to be determined.

The ML tree shows the sequences from the isolates and reference strains of cyanobacteria, in order to verify the diversity of the obtained isolates.

The phylogenetic tree consisted of 25 clusters (Figure 6), which were defined by being monophyletic groups with a bootstrap value of 70% or higher and had to include at least 1 isolate strain or 1 reference strain. An exception was made to the Cluster Y and X which had a bootstrap value lower than 50% but was defined as a cluster because it is a monophyletic group that includes the isolate strain. Clade N (Figure 6) includes PL1 and PL2, being supported by a high bootstrap value (100%) and is close related (bootstrap 94%) to *Lyngbya* cf. *confervoides* AY599507, which cannot be assigned as a reference strain for *Lyngbya*. Because of that, this clade is worthy to be described as a new cyanobacterial genus in further studies.

Clade S (Figure 6) also does not include a reference strain and is phylogenetically strongly supported (bootstrap 100%). It includes the isolated strain VV9 identified, which is phylogenetically close related to *Leptolyngbya* PCC7376 and the respective best hit that is an unidentified 4Oscillatoria_rosea_IAM. This clade is also worthy to be described as a new cyanobacterial genus in further studies.

The clade T is identified as *Jaaginema*, and includes the isolate obtained in this study JM1B the best hit strain to *Jaaginema* strain JaaginemasPsrJGgm 14 and JaaginemasPlkpSMP32 which are non-heterocyst filamentous, usually of very simple and thin morphology and without large distinctive characters [13] and one JQ013010_Filamentouscyanobacte.

The Clade X was subdivided into 2 subclades (Figure 6) subclade X1 included comprises 5 isolated strains (VV10,VV6,VV3A,VV3B,VR7A, VV3) all identified as belonging to not already described genera. The subclade X2 (figure 6) was integrated by 3 isolates strains VV13, JM1C and VV2 identified as a *Leptothoe*_TAU_MAC_161 and *Leptolyngbya*_sp_HBC2,it is important to refer that the VV13,JM1C and VV2 beside they are phylogenetically positioned in the same clade, their morphological characteristics are very similar even to they were collected from different places.

Clade Y (figure 6) comprised of 3 isolate strains VR7B,JM5A the Best hit strains *Nodosilinea* and *Leptolyngbya*_sp_LEGE_0 and VV1 the Best hit strains *Leptolyngbya*_sp_CENA15 and *Haloleptolyngbyaalcali* with a low bootstrap value (<50 %) this clade was only defined to illustrate the close proximity of the isolates.

In a general analysis we verified the presence of genera of common cyanobacteria in marine benthonic environments such as the genus *Leptolyngbya*. The most striking result was the possibility that most strains belong to new filamentous genera. In order support the 16S rRNA-based classification of cyanobacteria other molecular tools may be suggested such as the DNA-dependent RNA polymerase gamma subunit (rpoC1), and an in-depth morphological evaluation using electron microscopy. Being new genera, this work allows to contribute to the knowledge of cyanobacteria by revealing unreported cyanobacterial diversity.

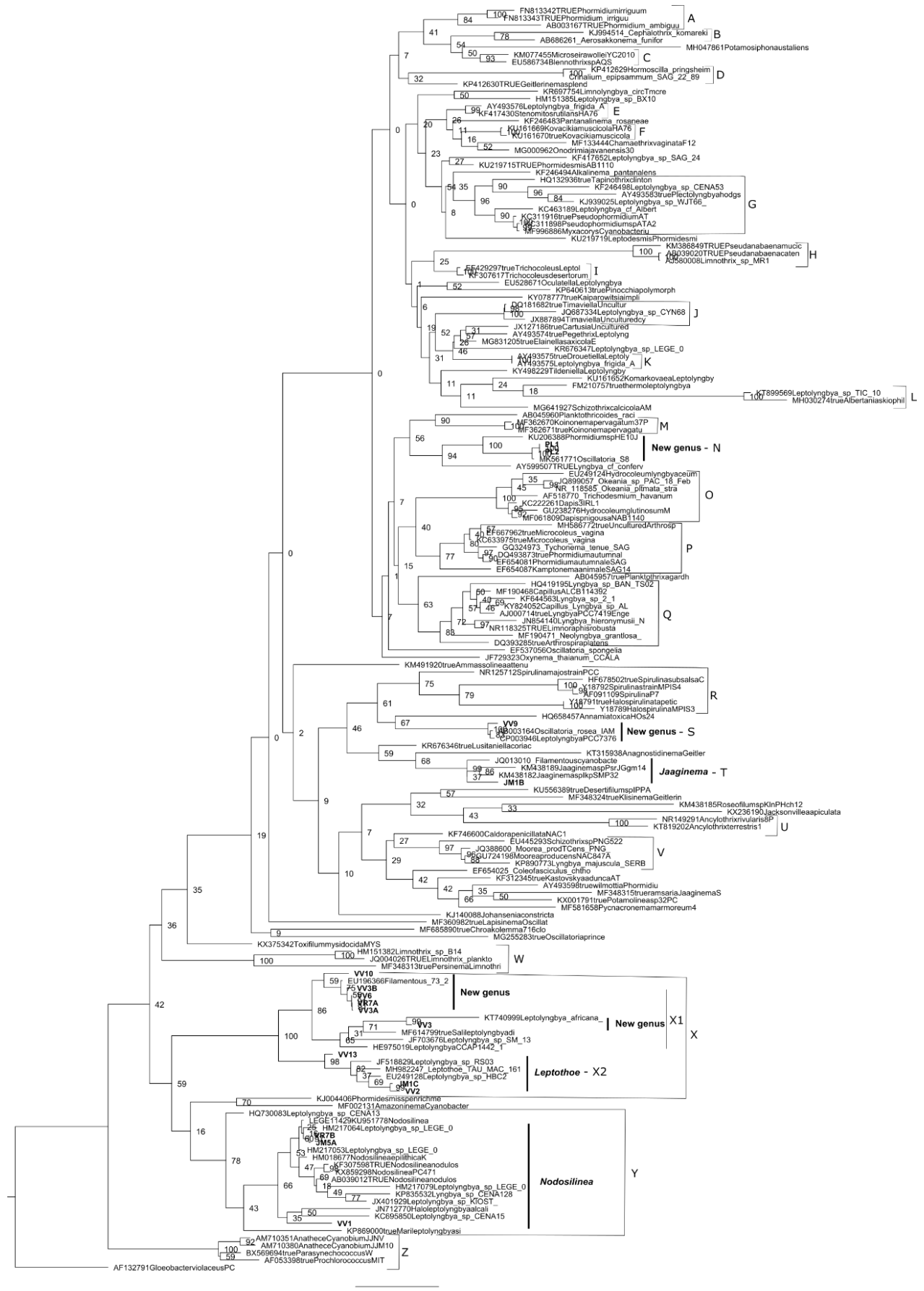


Figure 6 - Phylogenetic distribution based on the 16S rRNA of isolates (red) with the respective Best Hits.

4.3. Bioactivity

The potential bioactivity of the extracts was assayed against cancer and normal cell lines. For the evaluation of the anticancer potential, three cancer cell lines representative of major cancers affecting humans were selected, hepatic carcinoma cells HepG2, colon carcinoma cells RKO and breast adenocarcinoma cells T47D. Since the potential utility of the cyanobacteria extracts as anticancer drugs depends on the degree of selective toxicity on tumor cells, sparing normal cells, two normal cells were selected, the keratinocytes cell line HaCat and the endothelial cell line hCMEC/D3. The selection of these two normal cell lines was also based in the potential for skin care application that the cyanobacteria strains might revealed since keratinocytes are the major epidermis cells and endothelial cells are major blood vessels cells. The cytotoxicity of the cyanobacteria extracts was assayed by the MTT assay. The MTT assay is a widely used cytotoxic assay that measures cellular viability and thus, indirectly, cell proliferation. The assay was performed with the extract of the isolated strains at concentrations of $100\mu\text{g mL}^{-1}$; $75\mu\text{g mL}^{-1}$; $50\mu\text{g mL}^{-1}$; $25\mu\text{g mL}^{-1}$; $12.5\mu\text{g mL}^{-1}$ and $6\mu\text{g mL}^{-1}$ and exposure times of 24 and 48 hours. In all the assays the solvent control consisted in 1% DMSO in cell culture medium. Values are expressed as mean \pm SD, $n=3$. Results obtained are presented in Figures 6-18 and a summary is presented in Table 10.

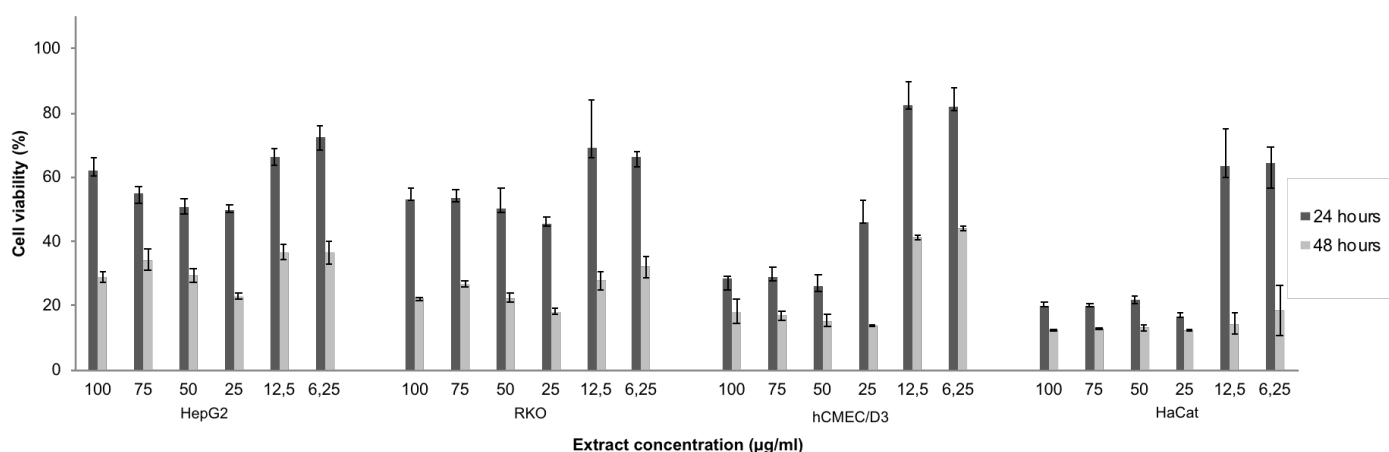


Figure 7 - Cellular viability of cells exposed to the extract of strain JM1C.

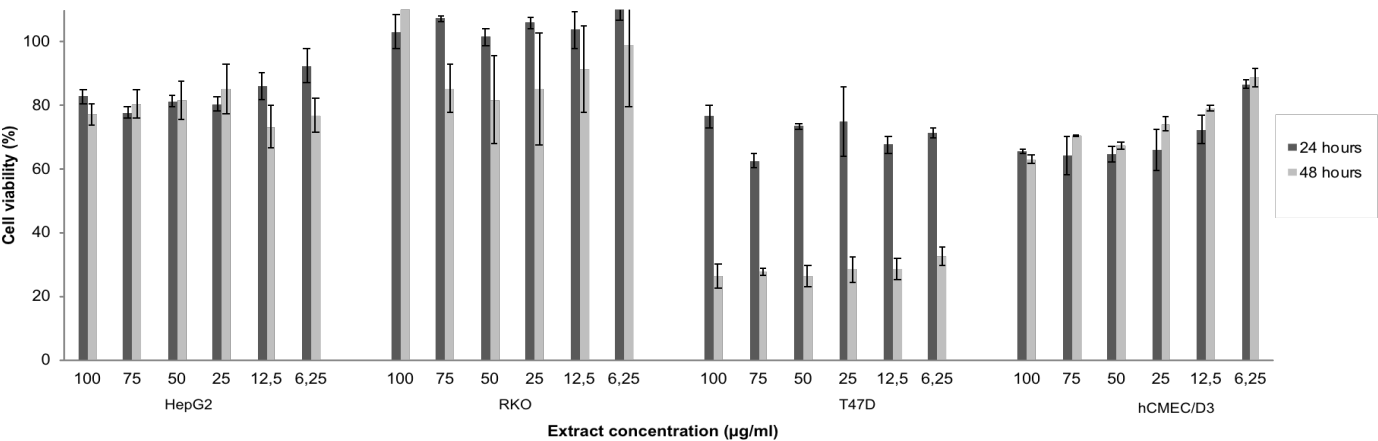


Figure 8 - Cellular viability of cells exposed to the extract of the strain JM1B.

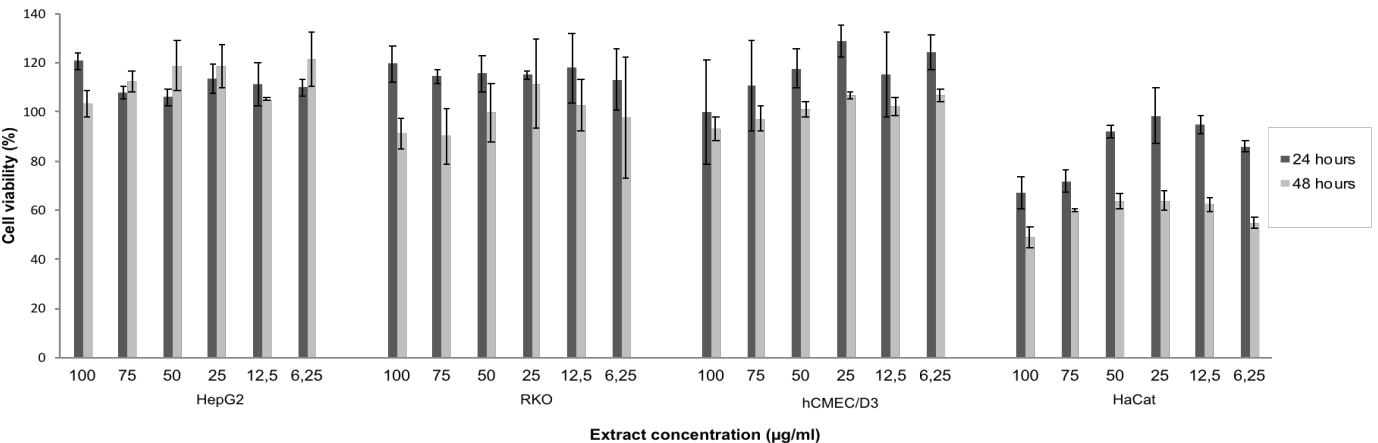


Figure 9 - Cellular viability of cells exposed to the extract of the strain PL1

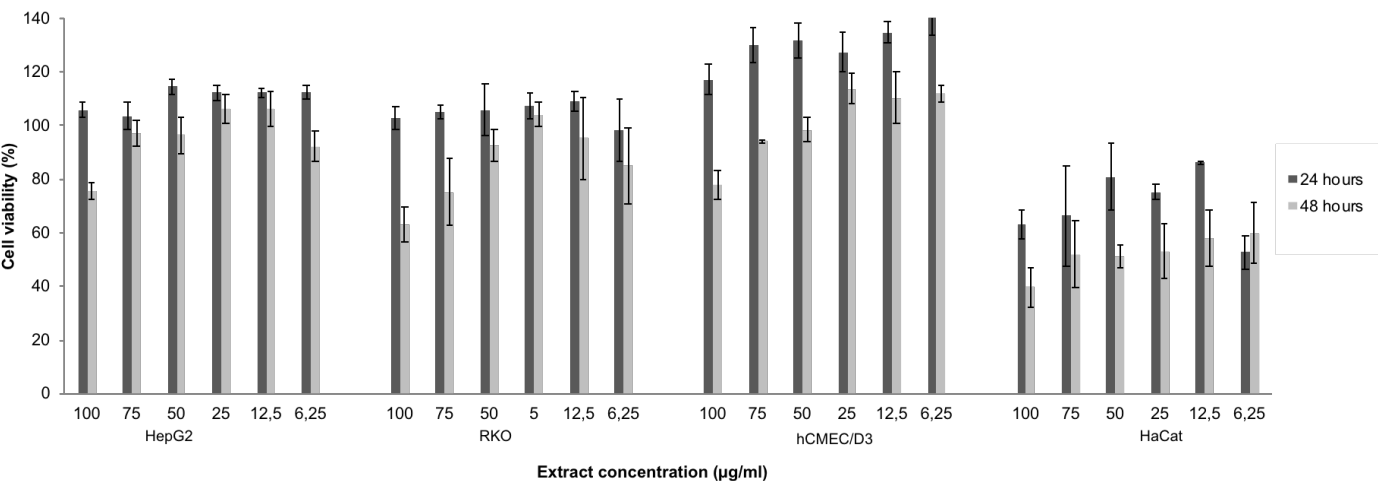


Figure 10 - Cellular viability of the cells exposed to the extract of the strain PL2

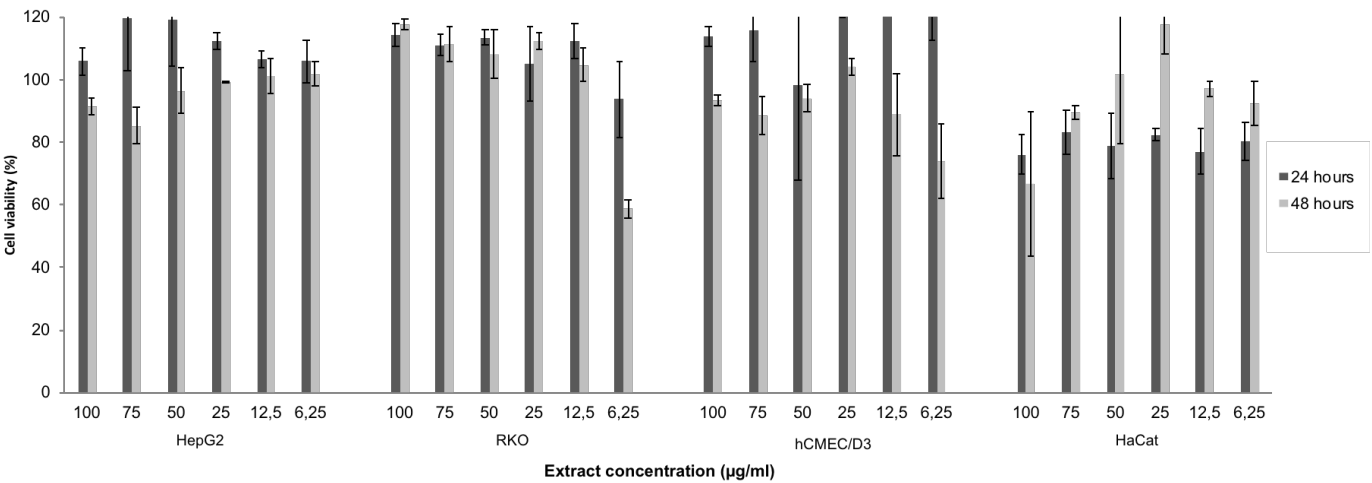


Figure 11 - Cellular viability of the cells exposed to the extract of the strain VV9

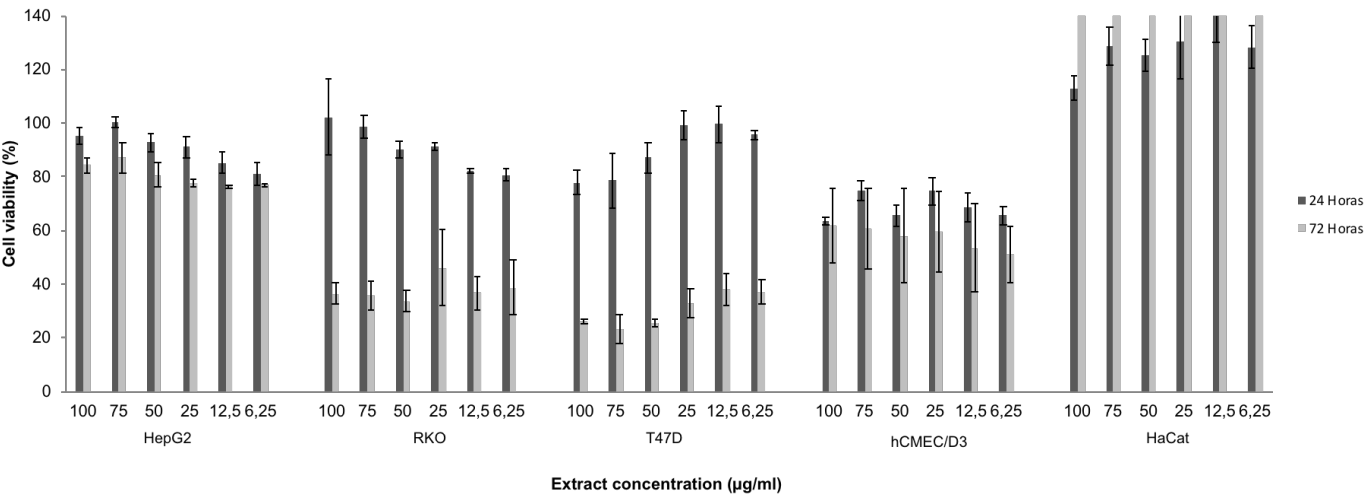


Figure 12 - Cellular viability of the cells exposed to the extract of the strain VV5

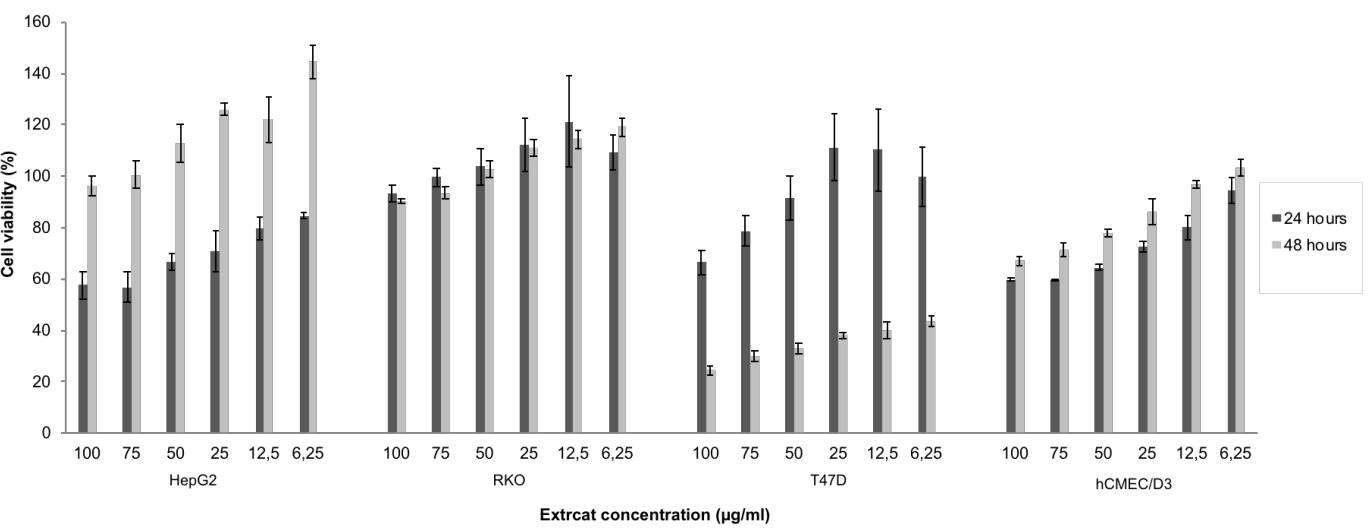


Figure 13 - Cellular viability of the cells exposed to the extract of the strain JM5A

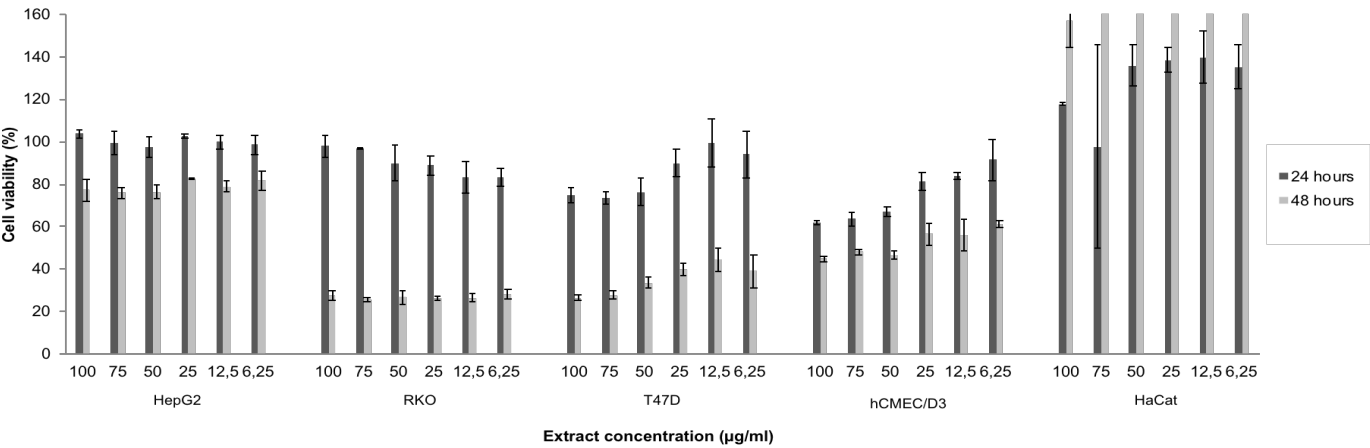


Figure 14 - Cellular viability of the cells exposed to the extract of the strain VV3

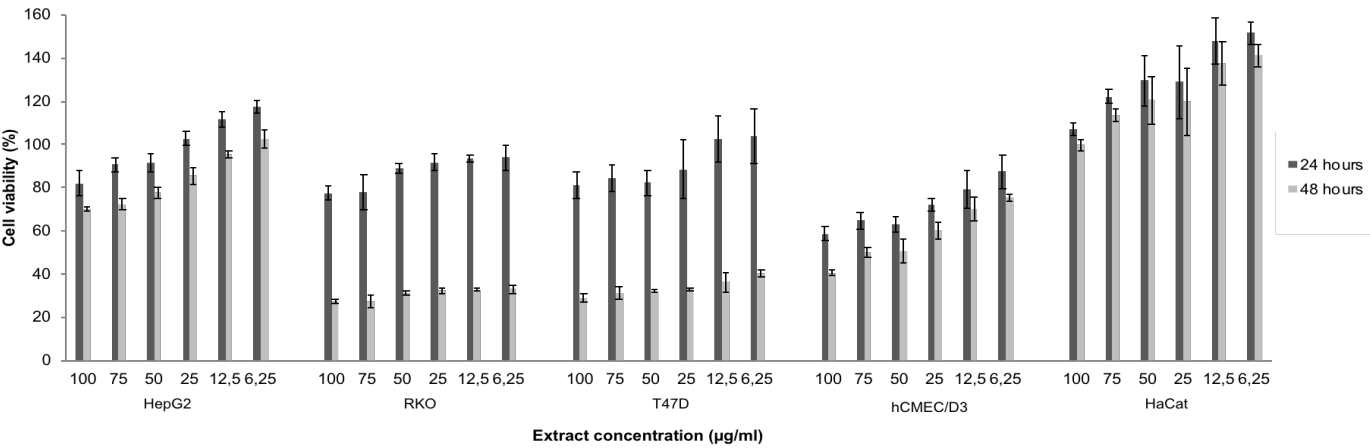


Figure 15 - Cellular viability of the cells exposed to the extract of the strain VV3B

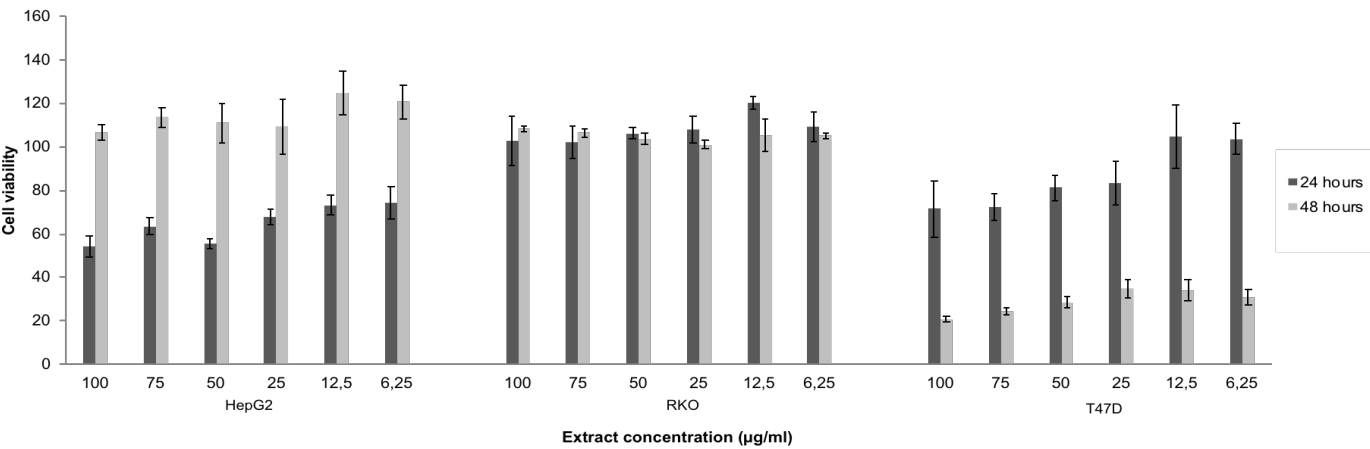


Figure 16 - Cellular viability of the cells exposed to the extract of the strain VR7A

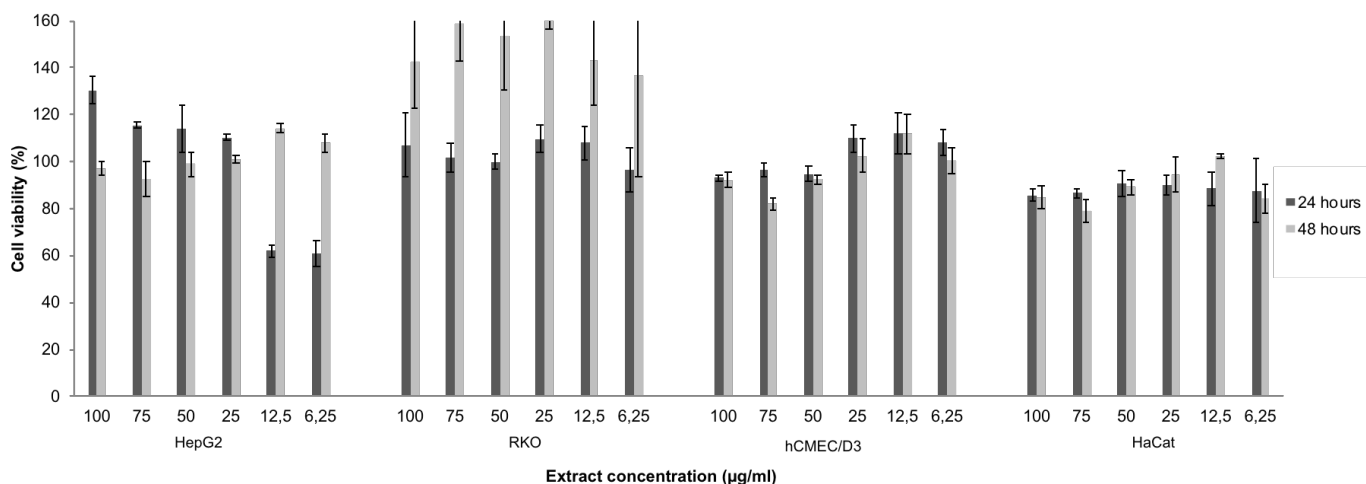


Figure 17 – Cellular viability of the cells exposed to the extract of the strain VR7B

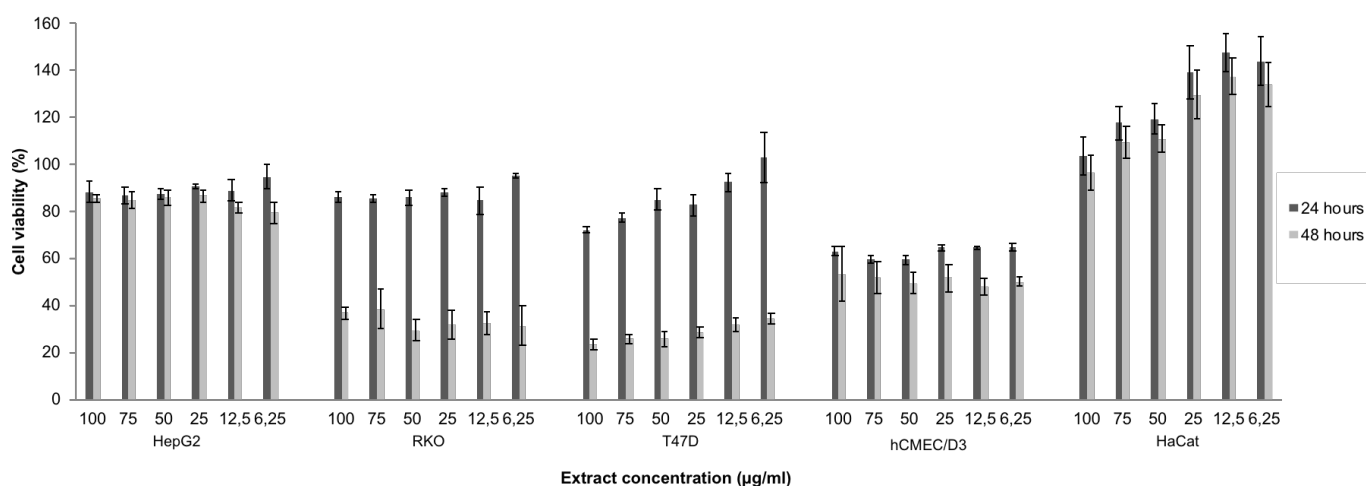





Figure 18 - Cellular viability of the cells exposed to the extract of the strain VV13

By analyzing the results, we can see that the results showed both a reduction and increase in cells viability. The strain JM1C was the most toxic one inducing a decrease in cell viability in all tested cell lines, especially after 48 hours incubation (Figure 7). This reduction was particularly evident in the endothelial cells and in the keratinocytes cell line. Although no selectivity between the different cell lines is evident, the cytotoxicity registered reflects the presence of bioactive compounds with potential anticancer effect. Since only one crude extract has been tested and it represents a mixture of compounds, this strain is interesting for the isolation of compounds which, once isolated, may exhibit differential bioactivity in different cell lines (colour in Table 10).


The strain JM1B is particularly interesting since different responses were obtained with the three different cancer cell lines. The extract of this strain induced a higher reduction of the cellular viability of the breast adenocarcinoma cell line T47D, especially after 48

hours incubation, with a reduction of nearly 70% in cell viability at all extract concentration (Figure 8). Although a reduction in cell viability of the normal endothelial cells hCMEC/D3 was also registered, this was between 30% and 40% for the $100 \mu\text{g mL}^{-1}$, $50 \mu\text{g mL}^{-1}$, $25 \mu\text{g mL}^{-1}$ and only 20% and 12% for the $12,5 \mu\text{g mL}^{-1}$, and $6,25 \mu\text{g mL}^{-1}$ concentrations respectively. The higher cytotoxicity of the extract to the T47D cell line in all concentrations compared to the slight cytotoxicity of the extract to the hCMEC/D3 cell line at the two lower concentrations tested, indicate the selectivity of the extract concerning the cancer cell line and the normal cell line. Also, the lower cytotoxicity registered for the hepatic carcinoma cell line HepG2 and the colon adenocarcinoma cell line RKO, indicates selectivity among cancer cell lines. This cyanobacteria strain is, in this study, one of the strains that further extract fractionation should be  formed in order to isolate bioactive compounds with potential anticancer interest ( colour in Table 10)..

The extract of the strain PL1 revealed no cytotoxicity for the cancer cell lines HepG2 and RKO and the normal cell line hCMEC/D3, however, a reduction between 50% and 40% was obtained with the keratinocytes cell line after 48 hours incubation (Figure 9). The same pattern was registered with the extract of the strain PL2 where only a slight decrease in cell viability at the concentrations higher than $25 \mu\text{g mL}^{-1}$ was registered in the cancer cell lines and a reduction of cell viability between 40-50% was obtained with the keratinocytes cell line (Figure 10). Although to a lesser extent, the results of cytotoxicity with the strain VV9 are similar with the two previous strains. This strain induced no toxicity or a slight toxicity against the cancer cell lines and the endothelial cells and a slightly higher cytotoxicity against the keratinocytes (Figure 11).

With the strain VV5, a reduction in cell viability, that reached 70%, was registered particularly after 48 hours incubation and especially for cells RKO and T47D (Figure 12). On the opposite, an increase in cell viability was registered for the keratinocytes HaCat. The same pattern was obtained with strains VV3 (Figure 14), VV3B (Figure 15) and VV13 (Figure 18). In addition to JM1C and JM1B these are also interesting strains to continuing for the isolation of bioactive compounds, in this case with anticancer potential and potential for the use on skin care products ( Colour in Table 10). Concerning keratinocytes these are the major constituents of the epidermis which is the superficial layer of the skin and the primary defense against exogenous factors such as UV radiation, pollution and cold [63]. In order to protect against these exogenous factors, skin regenerative actions occur, such as the proliferation of keratinocytes. The cyanobacteria strains that induced keratinocytes proliferation are thus interesting for the isolation of compounds with this potential. With these four cyanobacteria strains the

increase in keratinocytes viability was in fact evident. With the VV3 strain a percentage of cell viability up to 140% in the concentration of $100 \mu\text{g mL}^{-1}$, 120% at the concentration of $50 \mu\text{g mL}^{-1}$, $12,5 \mu\text{g mL}^{-1}$, $6,25 \mu\text{g mL}^{-1}$ and up to 80% in the concentration of $75 \mu\text{g mL}^{-1}$ for 24 hours after the exposure and for 48 the cell viability was up 160% for all concentrations was obtained (Figure 13). An increase of viability was also observed for a VV3B strain in the keratinocytes which obtained a percentage of cell viability up 100% in the concentration of $100 \mu\text{g mL}^{-1}$, 120% of $75 \mu\text{g mL}^{-1}$ and then up to 140% the concentration of $50 \mu\text{g mL}^{-1}$, $25 \mu\text{g mL}^{-1}$, $12,5 \mu\text{g mL}^{-1}$, and $6,25 \mu\text{g mL}^{-1}$ for 24 hours and which obtained a percentage of cell viability up to 80% at the concentration of $100 \mu\text{g mL}^{-1}$, $50 \mu\text{g mL}^{-1}$, $25 \mu\text{g mL}^{-1}$, 100% at the concentration of $75 \mu\text{g mL}^{-1}$ and then 120% at the concentration of $12,5 \mu\text{g mL}^{-1}$, $6,25 \mu\text{g mL}^{-1}$ (Figure 14) for 48 hours. For the VV5 strain as well in keratinocytes an increase of viability was obtained which at the concentration of $100 \mu\text{g mL}^{-1}$ and $50 \mu\text{g mL}^{-1}$ a percentage up to 100% was obtained and 120% at the concentration of $75 \mu\text{g mL}^{-1}$, $25 \mu\text{g mL}^{-1}$, $12,5 \mu\text{g mL}^{-1}$, $6,25 \mu\text{g mL}^{-1}$ for 24 hours and up to 140% for all the extract concentration after 48 hours (Figure 11). Even as VV3 strain we can see an increase of the viability of the cell after 48 hours of the exposure. For a VV13 strain also we can see that the highest the cell viability was observed in the keratinocytes which displayed a percentage of cell viability up to 100% at the concentration of $100 \mu\text{g mL}^{-1}$, 120% at the concentration of $75 \mu\text{g mL}^{-1}$, $50 \mu\text{g mL}^{-1}$, 160% at the concentration of $25 \mu\text{g mL}^{-1}$, $12,5 \mu\text{g mL}^{-1}$, $6,25 \mu\text{g mL}^{-1}$ for 24 hours and for 48 a percentage of cell viability up to 80% for the concentration $100 \mu\text{g mL}^{-1}$, up to 100% for the concentration of $75 \mu\text{g mL}^{-1}$, $50 \mu\text{g mL}^{-1}$ and up $25 \mu\text{g mL}^{-1}$, $12,5 \mu\text{g mL}^{-1}$, $6,25 \mu\text{g mL}^{-1}$ (Figure 17).

The cytotoxicity results obtained with the strains JM5A and VR7A were similar since the extracts affected more the T47D cells following the HepG2 cells and with no cytotoxicity against the RKO cells. As for strain JM1B, these results point to a selective toxicity of the extracts towards different cancer cell lines being thus also interesting for the isolation of potential anticancer compounds ( colour in Table 10).

Considering the endothelial cells hCMEC/D3, strains PL1, PL2 and VV9 induced cell proliferation (Figures 9, 10 and 11). This result is also interesting concerning skin care, since skin is a highly irrigated organ being irrigation closely linked to skin regeneration [64].

In general, there was some selectivity of the extracts, both in the whole of the cancerous lines and normal lines and between the normal lines and the cancerous lines. These results support the presence of compounds with bioactive activity that will be interesting to explore.

Table 10 - Summary of the cell viability results obtained with the MTT assay for each of the cyanobacteria strains and cell lines. +++ cell viability higher than 100%; ++ cell viability 100%-80%, + cell viability 80-70% and - cell viability lower than 70%.

[illegible]

5. Conclusions

The present work constituted a preliminary contribution for the study of the biodiversity and bioactive potential of cyanobacteria from Cape Verde Islands. From the collected samples 20 filamentous cyanobacteria strains were isolated. Since the cyanobacteria isolation process is very time consuming, it was not possible to isolate more strains within the time of this thesis. This isolation process is however being continued. The phylogenetic analysis reveals the possibility that strains belong to new genera, which contributes to enrich the diversity that occurs within this group of organisms.

Considering the bioactive assays performed using human cell lines it was possible to infer about the potential of some of the strains namely by the observed selective toxicity towards cancer cells and the induced proliferation of keratinocytes and endothelial cells. Although only a crude methanolic extract was tested, the bioactivity results are promising for the isolation of compounds with potential anticancer activity. Since only a crude extract, and therefore a cocktail of compounds was tested, fractionation becomes important. In this sense, the results obtained allowed a selection of strains which crude extract will be further fractionated in order to isolate the bioactive compounds.

Far from being completed this study represents the first approach to the study of the biodiversity and biotechnological potential of cyanobacteria from Cape Verde.

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